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MULTIPLE SHOOT CULTURES OF *RAUVOLFIA SERPENTINA*: GROWTH AND ALKALOID PRODUCTION

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ABSTRACT.—In vitro cultures were initiated from different parts of the plant *Rauvolfia serpentina*. Leaf and stem explants gave actively growing callus tissues on Murashige and Skoog (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (2 mg/liter)+ benzyladenine (BA) (1 mg/liter). Multiple shoots developed from axillary meristem explants on MS medium supplemented with BA in combination with naphthaleneacetic acid (NAA) or indoleacetic acid (IAA). Sustained growth of the shoot cultures was achieved on MS liquid medium containing (BA+NAA). The shoot cultures produced significantly high levels of alkaloids. One of the isolated alkaloids was characterized as 3-*epi*-α-yohimbine.

Multiple shoot cultures of medicinal plants are good sources of known and new pharmacologically active plant constituents (1,2). Unlike the whole plants, which are subject to changes due to environmental factors, the shoot cultures are grown under precise nutritional and growth conditions either in small vessels or in large bio-reactors (3,4). The shoot cultures, therefore, form a stable system for the production of high-value plant constituents. In our previous communication we have reported the presence of the indole alkaloids ajmaline, ajmalidine, and yohimbine in shoot cultures of *Rauvolfia serpentina* (L.) Benth. ex Kurz (Apocynaceae) (5). The present report concerns factors influencing the development of shoot cultures from selected explants of *R. serpentina* and the ability of the shoot cultures to produce alkaloids.

EXPERIMENTAL

PLANT MATERIAL AND TISSUE CULTURES.—Explant material was procured from a selected plant of *R. serpentina*, grown in Trombay Experimental field station, Bombay. Stem, nodal region, and leaf discs of one-year-old young plants were cut into 1-cm segments, sterilized, and incubated on (MS) medium (6), supplemented with different combinations of growth hormones (Table 1). The cultures were routinely transferred to fresh medium after 4 weeks of incubation. Callus cultures were maintained on agar medium and multiple shoot cultures in liquid medium.

CHEMICAL ANALYSIS.—Hplc was carried out on Waters Associate ALC/GPC-244, μ-Bondapak C_{18}

TABLE 1. Response of Stem, Leaf, and Axillary Meristem Explants of *Rauvolfia serpentina* to Phytohormones

Growth Hormones[a]	Axillary Bud	Stem	Leaf
MS+2,4-D(2)[b]	Slight callus formation from cut ends	Callus formation	No response
MS+NAA(2)	—	Slight callus formation	No response
MS+IAA(2)	Shoot bud development	No response	No response
MS+Kn(0.2)+2,4−D(2)	Slight callus formation from cut ends	Slight callus formation	Good callus formation
MS+BA(1)+2,4−D(2)	—	Good callus formation	Good callus formation
MS+IAA(2)+Kn(0.2)	Elongation of shoots buds	No response	No response
MS+IAA(2)+BA(1)	Shoot bud development	No response	No response
MS+NAA(2)+Kn(0.2)	Rooting	—	No response
MS+NAA(2)+BA(0.2)	Slight callus formation from cut end	Slight callus formation	Slight callus formation
MS+NAA(0.1)+BA(1.0)	Multiple shoot development	—	—
MS+Kn(1)+BA(0.2)	Development of axillary meristem	No response	No response

[a]2,4-D (2,4-dichlorophenoxyacetic acid); NAA (naphthaleneacetic acid); IAA (indoleacetic acid); Kn (kinetin); BA (benzyladenine).
[b]mg/liter.

using uv detector set at 254 nm and solvent system MeOH-MeCN-potassium phosphate buffer (pH 8.0) (40:20:40) with a flow rate of 1 ml/min. Optical rotations were recorded on Perkin-Elmer 141 polarimeter, nmr on Varian A-10, and ms on V.G. Micromass 7070 H.

Shoot cultures as well as roots were extracted, and basic fractions were separated by the method of Timmins and Court (7). Column chromatography (Si gel, $CHCl_3$/MeOH gradient) of the strongly basic fraction followed by medium pressure liquid chromatography (Si gel<0.08 mm, $CHCl_3$/MeOH gradient, 50 psi), and preparative tlc (Si gel C) yielded ajmaline, yohimbine, and two new compounds (I and II).

COMPOUND 1.—Pale yellow amorphous powder, mp 125° (softening observed at 105°), $[\alpha]_D - 105°$ (pyridine), uv λ max (MeOH) nm 278, 290 [sh], 225; ms *m/z* 354 (M^+,20%), 353 ($M^{\pm}1$, 100%), 339 (17%), 323 (10%), 295 (15%), 277 (5%), 265 (5%), 251 (5%), 237 (5%), 223 (15%), 209 (10%), 197 (15%), 184 (40%), 169 (70%), 156 (40%), 144 (40%), 83 (30%); ir ν max (KBr) cm^{-1} 3400, 2950, 2875 (sh), 1720, 1450, 1200, 1150, 1100, 1055, 1000, 925; ^1H nmr (60 MHz, $CDCl_3$) δ 1.26 (b,m), 1.68, 2.16 (b,m), 2.56, 2.78, 3.2, 3.6 (b,m,1p), 3.86 (4p), 4.44 (b,m,1p), 6.86, 7.3 (aromatic protons), and 7.84 (-NH, 1p).

COMPOUND 2.—Uv λ max (MeOH) nm 290, 248; ms *m/z* 326 (M^+,100%), 311 (30%), 183 (43%); both identical with those of ajmaline and same color reaction as ajmaline with $FeCl_3/HClO_4$ spray. The Rf value of the compound (0.32) was more than ajmaline (0.26). Further studies towards its identity are in progress.

The weakly basic fraction, upon chromatographic separation, yielded ajmalidine.

RESULTS AND DISCUSSION

TISSUE CULTURES.—The axillary meristem of the nodal explants developed shoots on MS medium supplemented with benzyladenine (BA) as one of the growth hormones. In agar medium containing BA+naphthaleneacetic acid (NAA), multiple shoots developed from the basal end of the explant without the intervention of callus mass (Figure 1a). These shoots could be propagated successfully in MS liquid medium of the same composition (Figure 1b). The meristem explants did not respond well in medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), and slight callus formation was observed at the cut ends. In medium containing NAA+Kinetin (Kn) combinations, shoot development followed by profuse rooting was observed from the cut end of the explant (Figure 1c). The roots could be isolated and grown in NAA+Kn liquid medium. The leaf and stem explants gave callus tissues in medium supplemented with different combinations of 2,4-D, Kn, and BA. Sustained growth of the callus was achieved in medium containing 2,4-D+BA combination. The response of the tissue to different growth hormones is summarized in Table 1.

The multiple shoot cultures have been maintained in liquid medium for prolonged periods through periodic subcultures of 30 days. The shoot cultures grew uniformly during the first 4 weeks and showed maximum growth during the 4th and 5th weeks. The cultures have shown consistency in their growth pattern and morphogenetic response over a period of 5 years.

ALKALOIDS.—The uv, ms, and tlc data of compound 1 indicated that it could be an isomer of yohimbine. Seven optically active forms of yohimbine are known to occur in nature all having an α-axial proton at C-15 (8). The negative molecular rotation (−105°) and mp (125°) of the compound suggested that it could be 3-*epi*-α-yohimbine (9,10). Further support for the identity of the compound was obtained through its ir and nmr spectral data. The ir spectrum of the compound showed the absence of any distinct peaks in the region 2700-2900 cm^{-1} and a slight shoulder on the longer wave length side of the major peak (2950 cm^{-1}). These data indicated an equatorial β orientation for the C-3-H (11,12). The nmr signal for one proton at δ 4.44 further supported the equatorial configuration of the C-3 proton. An axial proton would give a signal at a higher field (13). These data confirmed the identity of the compound 1 as 3-*epi*-α-yohimbine.

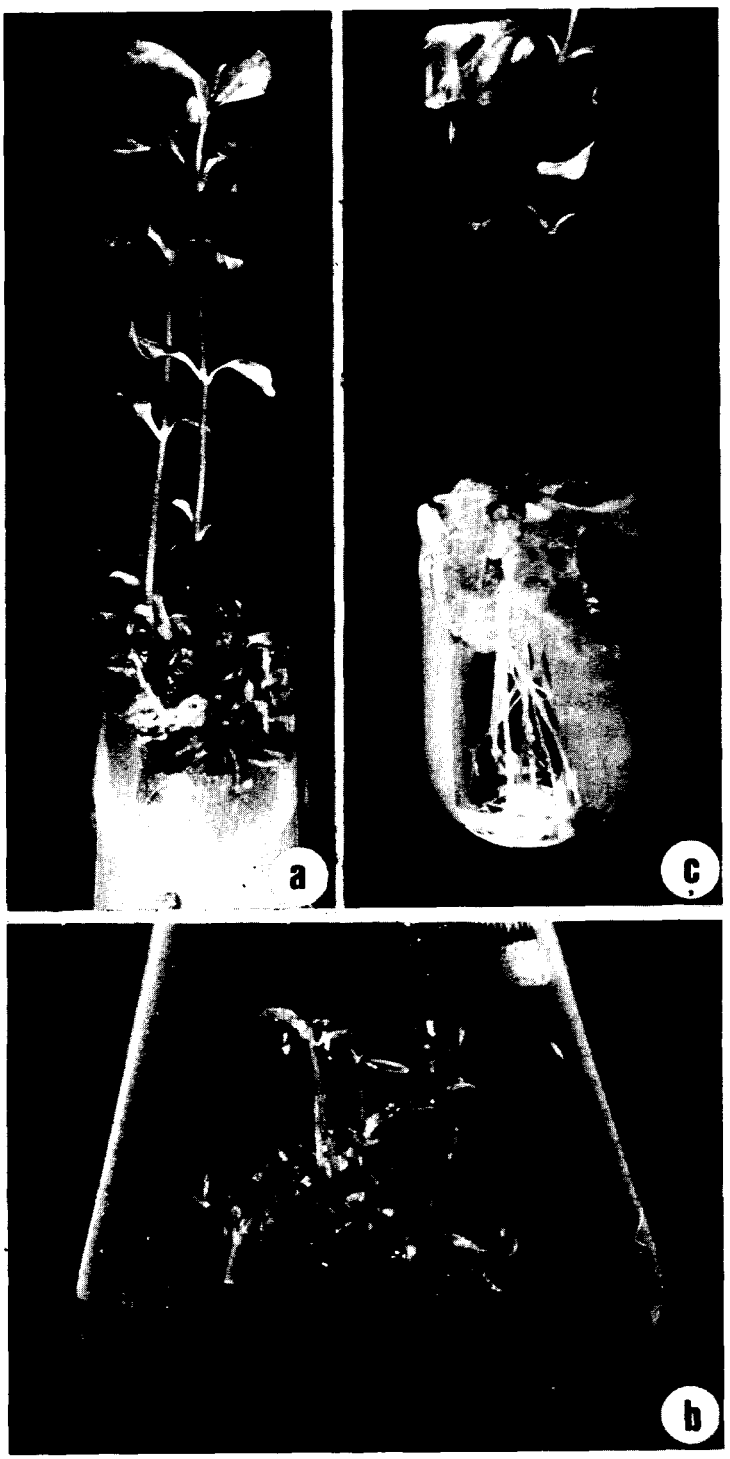

FIGURE 1. Response of axillary meristem explant to different growth hormones: a) shoot development in BA (1 mg/liter)+NAA (0.1 mg/liter) medium, b) multiple shoot formation in MS liquid medium with BA (1 mg/liter)+NAA (0.1 mg/liter), c) shoot development and profuse rooting in NAA (2 mg/liter)+Kn (0.2 mg/liter)

Hplc analysis of the total alkaloids from the roots, young shoots, and shoot cultures revealed that these tissues contained similar levels of yohimbine and 3-*epi*-α-yohimbine. The roots contained the highest level of ajmaline, followed by the shoot cultures and the tender shoots. Ajmalidine was present in the leaves and shoot cultures but was absent in the roots (Table 2). The major alkaloids of the roots, reserpine and rescinnamine, were found in trace amounts in the shoot cultures. The shoot cultures have been producing consistently high levels of alkaloids for more than 5 years. The alkaloid composition and concentration have not undergone major changes during this period.

TABLE 2. Alkaloid Content in Roots, Leaves, and Shoot Cultures of *Rauvolfia serpentina*[a]

Material	Ajmalidine[b]	Yohimbine	3-*epi*-α-Yohimbine	Ajmaline	Total alkaloid content by weight, strongly basic fraction
Roots	—	0.011	0.029	0.094	1.28
Leaves	0.013	0.008	0.031	0.013	0.63
Shoot cultures[c]	0.018	0.007	0.040	0.021	0.88

[a]In percent dry weight.
[b]Ajmalidine content was measured using yohimbine standard curve.
[c]Reserpine and rescinnamine were found in trace amounts.

The morphogenetic response of the axillary meristem such as rooting with NAA+Kn and multiple shoot formation with BA+NAA indicated that in vitro methods would be useful in cloning elite varieties of *Rauvolfia*. The consistency in the morphogenetic response and the biosynthetic pattern of the shoot culture could be attributed to the fact that the cultures originated without the intervention of unorganized callus. It is, therefore, reasonable to expect that the plants developed from the cultures would also exhibit the stem lineage.

It is of interest to note that the shoot cultures gave good yields of biomass and synthesized substantial amounts of total alkaloids in a period of 35 days. The shoot cultures contained alkaloids found in the roots as well as the shoots of the intact plant. The cultures, therefore, represent the metabolic pattern of the whole plant to some extent.

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